AD_____

Award Number: DAMD17-98-1-8063

TITLE: Role of Cell Senescence in Breast Cancer

PRINCIPAL INVESTIGATOR: Ana Krtolica, Ph.D.

CONTRACTING ORGANIZATION: Lawrence Berkeley National Laboratory
Berkeley, California 94720

REPORT DATE: August 2001

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	August 2001	Annual Summary (1 Aug 98 - 31 Jul 01)

4. TITLE AND SUBTITLE
Role of Cell Senescence in Breast Cancer

5. FUNDING NUMBERS
DAMD17-98-1-8063

6. AUTHOR(S)
Ana Krtolica, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Lawrence Berkeley National Laboratory
Berkeley, California 94720

E-Mail: akrtolica@lbl.gov

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Report contains color

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

Cancer incidence rises exponentially with age. This project tested the hypothesis that cellular senescence of stromal fibroblasts contributes to the age-dependent increase in breast cancer by creating a more permissive environment for the expression of malignant phenotypes by breast epithelial cells.

Here, we report that senescent human fibroblasts stimulate premalignant and malignant, but not normal, breast epithelial cells to proliferate in culture and form tumors in mice. In culture, the growth stimulation was evident when senescent cells comprised only 10% of the fibroblast population, and was equally robust whether senescence was induced by replicative exhaustion, oncogenic RAS, p14ARF or hydrogen peroxide. Moreover, it was due at least in part to soluble and insoluble factors secreted by senescent cells. In mice, senescent, much more than presenescent, fibroblasts caused premalignant and malignant epithelial cells to form tumors. Taken together, these results support our hypothesis that cellular senescence of stromal fibroblasts may contribute to breast tumorigenesis.

14. SUBJECT TERMS
Breast Cancer, Cellular Senescence, Tumor microenvironment, Tumor promotion and progression, Co-culture

15. NUMBER OF PAGES
46

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Cover.....1

SF 298.....2

Table of Contents.....3

Introduction.....4

Body.....5

Key Research Accomplishments.....11

Reportable Outcomes.....12

Conclusions.....13

References.....14

Appendices.....15

Final Report

Role of Senescence in Breast Cancer

Introduction

Age is the main risk factor for the majority of human cancers, including breast cancer. The goal of this project was to investigate whether cellular senescence contributes to this age-related rise in cancer frequency. Specifically, we wanted to test the hypothesis that cellular senescence of stromal fibroblasts alters the environment of breast epithelial cells such that it becomes permissive for the expression of malignant phenotypes. With this in mind, we created cell culture systems that allowed us to explore the influence of senescent fibroblasts on breast epithelial cell behavior. In particular, we developed direct and indirect co-culture systems and methods to quantify changes in breast epithelial cell proliferation under these conditions. In addition, we confirmed some of the results observed in our *in vitro* co-culture system by *in vivo* tumorigenicity studies in nude mice. Our studies have revealed one of the mechanisms by which the aging tissue environment can contribute to age-related breast cancer development.

Body of the Final Report

Senescent human fibroblasts do not stimulate invasiveness of breast epithelial cells. To test the effect of soluble factors produced by senescent fibroblasts on the ability of breast epithelial cells to invade the stroma we performed the following experiments. Presenescent or senescent fibroblasts were seeded as lawns onto the lower wells of a modified Boyden invasion chamber (Transwell, Costar)(1). The ability of non-invasive S1 or invasive MB MDA453 breast epithelial cells to traverse the porous membrane coated with Matrigel (basement membrane extract) in the presence or absence of fibroblasts in the lower well was counted microscopically, as previously described (1). There was no significant difference in invasiveness in the presence of senescent compared to presenescent breast fibroblasts for both breast cell lines examined.

Senescent fibroblasts stimulate preneoplastic epithelial cell growth. We used equal numbers of presenescent and senescent cells to produce 50-80% confluent lawns onto which epithelial cells were seeded. We used four epithelial cell lines. S1 human mammary epithelial cells (2), HaCAT human epidermal keratinocytes (3) and SCp2 mouse mammary epithelial cells (4) are immortal, harboring p53 mutations, but do not form tumors in immunocompromised mice (2, 3, 5)(see Appendix). Thus, they are preneoplastic, having acquired only some mutations that predispose to malignancy. In addition, we used MDA231, an aggressive human breast cancer cell line (6). We also used normal human mammary epithelial cells from adult and human keratinocytes from adult or neonatal donors. Like the fibroblasts, these normal strains have a finite replicative capacity and no known mutations that predispose to malignancy. We preincubated epithelial cells in growth-factor-deficient medium before seeding them in this medium onto fibroblast lawns.

We first evaluated preneoplastic and neoplastic epithelial cells co-cultured with fibroblasts using Rhodanile Blue, which preferentially stains epithelial colonies (Fig. 1A). Senescent fibroblasts, much more than presenescent fibroblasts, stimulated the growth of all four cell lines. To quantify this, we stained the co-cultures with DAPI, a fluorescent DNA dye, and quantified the smaller, more intensely stained epithelial nuclei by image analysis (Fig. 1B). Alternatively, we expressed enhanced green fluorescent protein (EGFP) in the epithelial cells, and measured EGFP fluorescence. Compared to presenescent fibroblasts, senescent fibroblasts stimulated HaCAT, S1 and MDA231 cells 2- to 4-fold, and SCp2 cells 3- to 7-fold (Fig. 1B).

This differential growth stimulation was seen with three human fibroblast strains: WI-38 (fetal lung), 82-6 (adult skin), and 184 (adult breast).

Cells expressing a senescence marker (7) accumulate with age (7, 8, 9), but remain relatively rare, even in old tissues. To determine whether senescent fibroblasts stimulate preneoplastic epithelial cells even when abundant presenescent fibroblasts are present, we cultured SCp2 cells alone or on lawns containing varying fractions of senescent fibroblasts, and stained with Rhodanile (Fig. 1C) or DAPI (Fig. 1D) 8 d later. SCp2 proliferation was minimal in the absence of fibroblasts (Fig. 1C, panel *i*; 1D, No Fb). Presenescent fibroblasts stimulated growth 5-8 fold (Fig. 1C, panel *ii*; 1D, 10:0), which could be caused by presenescent fibroblasts *per se*, or by the 10-30% senescent cells always present in presenescent cultures. Whatever the case, increasing proportions of senescent fibroblasts progressively stimulated additional SCp2 growth, even when senescent cultures were only 10% of the fibroblast population (Figs. 1C-D). This experiment also suggests that presenescent fibroblasts do not inhibit the growth of preneoplastic epithelial cells, but, rather, that senescent cells facilitate their growth.

Senescent fibroblasts do not stimulate normal epithelial cells. In striking contrast to preneoplastic and neoplastic epithelial cells, genetically normal keratinocytes grew equally well on presenescent and senescent fibroblasts. After 8 d in co-culture, there was no statistical difference between growth on presenescent and senescent lawns. This was true for neonatal and adult human keratinocytes, and human mammary epithelial cells. Thus, although senescent fibroblasts stimulated preneoplastic and malignant epithelial cells, they did not differentially stimulate normal epithelial cells.

Kinetics. To follow the kinetics of the growth stimulation, we seeded EGFP-expressing HaCAT cells onto presenescent or senescent fibroblast lawns, and monitored EGFP fluorescence with time (Fig. 2A). Growth on senescent fibroblasts surpassed that on presenescent fibroblasts within 4 d (Fig. 2A), and continued to do so until the epithelial cells reached confluence.

Contribution of secreted factors. Senescent fibroblasts might stimulate epithelial cell growth by direct cell-cell interaction, or by secreting diffusible factors or an insoluble extracellular matrix.

To test these possibilities, we cultured cells in two-chamber dishes. These separated fibroblast lawns from the epithelial cells by a porous membrane, preventing direct contact but

permitting exchange of soluble diffusible factors. Soluble factors secreted by senescent fibroblasts were 2- to 3-fold more potent in stimulating SCp2 (Fig. 2B) growth than those secreted by presenescent fibroblasts. Overall, however, senescent fibroblast-derived soluble factors were 10-fold less potent than direct cell contact (Fig. 2B, Soluble vs Cells, black bars), even though epithelial cells attached equally well to the membrane and fibroblasts.

To determine the contribution of secreted matrices, we allowed fibroblasts in serum-free medium to deposit extracellular matrix onto culture dishes for 2-3 days. We then removed the cells by calcium chelation or mild detergent. We plated SCp2 cells onto the matrices, and quantified cell number by DAPI fluorescence. Matrix produced by senescent fibroblasts was 3- to 4-fold more stimulatory than matrix produced by presenescent fibroblasts (Fig. 2B). This difference was not due to differences in epithelial cell attachment.

Together, these results indicate that about 10% of the growth stimulation caused by senescent fibroblasts was due to secreted soluble factors, while 40% was due to secreted extracellular matrix. These are minimal estimates because cells may experience higher levels of soluble factors in direct co-culture, and/or matrix components may be lost or inactivated during cell removal. Thus, at least 50% of the growth stimulation was attributable to the secretory phenotype of senescent fibroblasts.

Growth stimulation is independent of the senescence inducer. Overexpression of certain oncogenes or tumor suppressor genes or DNA damage can induce a phenotype that closely resembles replicative senescence (10, 11). We therefore asked to what extent the ability to stimulate preneoplastic epithelial cells depended on replicative exhaustion.

We induced senescence by overexpressing p14^{ARF} or oncogenic RAS [RAS-Ha(V12)] (12) or by treatment of fibroblasts with a sublethal dose of H_2O_2 (13, 14)(see Appendix). We seeded HaCAT cells onto lawns of presenescent or senescence-induced fibroblasts and assessed HaCAT number by EGFP fluorescence.

Fibroblasts induced to senesce by several means stimulated the proliferation of preneoplastic epithelial cells independent of the inducer. This stimulation was 50-70% of that

Fig. 1. Effects on preneoplastic and malignant epithelial cells. Epithelial cells were plated on WI-38 fibroblast lawns, and cultured in growth factor-deficient medium for 8 d.

A. S1 (*i, iv*), SCp2 (*ii, v*) and HaCAT (*iii, vi*) cells, cultured on presenescent (*i-iii*) or senescent (*iv-vi*) lawns, stained with Rhodanile (240X).

B. Co-cultures described in (A), and co-culture with MDA231 cells, stained with DAPI. Epithelial nuclei were quantified as described in Methods. The results (in arbitrary units) shown are from 1 of 3-5 experiments. Error bars = standard error of the means (SEM) of duplicate or triplicate wells. Gray bars, presenescent lawns; black bars, senescent lawns.

C. SCp2 cells cultured without fibroblasts (*i*), or with presenescent and senescent cultures at ratios 10:0 (*ii*), 9:1 (*iii*), 8:2 (*iv*), 5:5 (*v*), and 0:10 (*vi*), stained with Rhodanile (240X).

D. SCp2 cells cultured without fibroblasts (No Fb) or with ratios of fibroblasts described in (C), quantified by DAPI fluorescence. Error bars = SEM from triplicate wells from 1 of 2 experiments.

Fig. 2. Characteristics of the growth stimulation.

A. EGFP-expressing HaCAT cells, seeded onto presenescent or senescent WI-38 lawns. EGFP fluorescence (in arbitrary units) was measured after 1-8 d, as indicated. Error bars = SEM from duplicate wells from 1 of 2 experiments. Gray bars, presenescent lawns; black bars, senescent lawns.

B. SCp2 cells, seeded onto WI-38 fibroblasts (Cells), plated in the upper chambers of Millicells containing fibroblasts in the lower chambers (Soluble), or plated onto matrices deposited by fibroblasts (Matrix), as described in Methods. Cell number was assessed after 8 d by DAPI fluorescence. Error bars = SEM from duplicate wells from 1 of 2 experiments. Gray bars, presenescent fibroblasts; black bars, senescent fibroblasts.

C. HaCAT cells, seeded onto lawns of control (left) or p14*ARF*-expressing (right) presenescent 82-6 fibroblasts, stained 8 d later with Rhodanile (250X).

D. HaCAT cells, quantified by EGFP fluorescence after co-culture with control (–) or p14*ARF*-expressing (+) 82-6 fibroblasts (ARF); control (–) or p14*ARF*-expressing (+) hTERT-immortalized 82-6 cells (TERT-ARF); control (–) or RAS-Ha/V12-expressing (+) WI-38 cells (RAS); 82-6 cells untreated (–) or treated (+) with H_2O_2; or replicatively senescent WI-38 cells (R). Growth was assessed after 8 d, except for RAS panels, where growth was assessed after 5 d. Error bars = SEM from duplicate wells from 1 of 2 experiments. Gray bars, presenescent lawns; black bars, senescent lawns.

caused by replicatively senescent fibroblasts, indicating that, at least qualitatively, the growth stimulation was independent of the senescence inducer (Fig. 2C and D).

Senescent fibroblasts stimulate mammary tumorigenesis. To test the idea that senescent cells create a microenvironment that promotes the growth of potentially or frankly neoplastic cells *in vivo*, we injected epithelial cells, alone or with fibroblasts, into immunocompromised (*nu/nu*) mice (14)(see Appendix). These experiments were not presented in our original Statement of Work, but were mentioned as a possible next phase if the proposed *in vitro* experiments were accomplished and supported our hypothesis. We did not anticipate that we will reach this phase before the end of the fellowship. However, since *in vitro* experiments were advancing faster than predicted and showed very strong effects, we decided to proceed with this next phase immediately and test whether the *in vivo* results will support our culture data. All the procedures were done according to an approved animal protocol.

SCp2 cells alone did not form tumors (14)(see Appendix), as reported (5). Moreover, when injected with presenescent fibroblasts, they did not form tumors after 160 d. However, when injected with senescent fibroblasts, they formed very large (400-2000 mm^3) tumors in 4/6 animals (14)(see Appendix). Thus, senescent fibroblasts stimulated hyperproliferation and neoplastic progression of non-tumorigenic mammary epithelial cells *in vivo*.

Unlike SCp2 cells, MDA231 are highly tumorigenic breast cancer cell line. Small numbers (2×10^5) of MDA231 cells produced tumors that reached 300-400 mm^3 in 2/5 mice in 45 d. hTERT-immortalized fibroblasts slightly accelerated tumorigenesis, causing tumors that reached 100-500 mm^3 in 4/5 mice. Senescent fibroblasts greatly accelerated tumorigenesis. Four of five mice developed tumors that reached 300-1600 mm^3 (14)(see Appendix). Thus, senescent fibroblasts strongly stimulated or facilitated tumorigenesis of both non-tumorigenic and tumorigenic breast epithelial cells.

Key Research Accomplishments

1. Senescent human fibroblasts stimulate 2-7 fold pre-neoplastic and neoplastic breast epithelial cell proliferation compared to presenescent fibroblasts.

2. Senescent fibroblasts do not stimulate more than presenescent fibroblasts normal human keratinocyte and mammary epithelial cell growth.

3. Senescent fibroblast-derived extracellular matrix contributes 40% and soluble factors 10% to the increase in breast epithelial cell proliferation.

4. The effect of senescent fibroblasts on breast epithelial cell proliferation is observable even in a mixed pre- and senescent fibroblast population. There is 50-100% increase in growth with only 10% senescent fibroblasts present.

5. Fibroblasts induced to senesce by $p14^{ARF}$ or oncogenic ras expression or by treatment of fibroblasts with a sublethal dose of H_2O_2, stimulate epithelial cell proliferation.

6. Senescent fibroblasts stimulate mammary tumorigenesis both of non-tumorigenic and tumorigenic breast epithelial cell lines.

7. Senescent human fibroblasts do not stimulate invasiveness of breast epithelial cells.

Reportable Outcomes

- Krtolica, A., S. Parrinello, S. Lockett, P.-Y. Desprez and J. Campisi. *"Senescent Fibroblasts Promote Epithelial Cell Growth and Tumorigenesis, Linking Cancer and Aging."* Proceedings National Academy of Sciences, *in press.*

- Invited Seminar Presentation: University of California San Francisco, San Francisco, CA: *"Models for studying role of altered stroma in tumorigenesis."* February 2001.

- Invited Seminar Presentation: National Cancer Institute, NIH, Bethesda, Maryland: *"Pay-off time: cellular senescence stimulates tumorigenesis."* October 2000.

- Poster presentation and Young Investigator Award at American Association for Cancer Research Annual Meeting. April 2000. San Francisco, CA. Krtolica, A., D. Yip, G. Dimri, P. Y. Desprez, and J. Campisi. *"The Double-edged Sword of Replicative Senescence: Senescent Fibroblasts Stimulate Pre-malignant Epithelial Cell Growth."*

- Krtolica, A, C. Ortiz, S. Lockett and J. Campisi. *"A fluorescence-based image analysis method for quantifying epithelial cells in co-culture with fibroblasts." Manuscript in preparation.*

- Applied for DOD BCRP IDEA and Career Development Awards: *"Aging, mutations and breast carcinogenesis".*

Conclusions

Our research addressed an important, but very much understudied, area of the pathogenesis of breast cancer: the role of aging and damaged stroma in cancer promotion and progression. Our findings indicate that the accumulation of senescent stromal cells with age (due to replicative exhaustion and/or damage) may create a pro-carcinogenic microenvironment that promotes breast cancer development. Therefore, we suggest a new link between cancer and aging, and a plausible mechanism by which genetic (oncogenic mutations) and epigenetic (accumulation of senescent cells) events synergize to generate the exponential rise in cancer that occurs with aging.

In the long term, understanding the role changes in tissue microenvironment, such as accumulation of damaged and senescent cells during aging, may have in breast carcinogenesis is crucial for developing rational preventive and intervention strategies. For example, decisions on how to treat ductal carcinoma *in situ* may be influenced by the extent to which the surrounding breast stroma is modified. In addition, it is not beyond the realm of possibility to envision strategies aimed at selectively reversing or preventing changes in the stromal microenvironment caused by senescent cells.

References:

1. Krtolica A, Ludlow JW. (1996) Hypoxia Arrests Ovarian Carcinoma Cell Cycle Progression, but Invasion is Unaffected. *Cancer Res.* **56**:1168-1173.
2. Briand P, Petersen OW, van Deurs B. (1987) A new diploid nontumorigenic human breast epithelial cell line isolated and propagated in chemically defined medium. *In Vitro Cell Dev. Biol.* **23**:181-188.
3. Skobe M, Fusenig NE. (1998) Tumorigenic conversion of immortal human keratinocytes through stromal cell activation. *Proc. Natl. Acad. Sci. U.S.A.* **95**:1050-1055.
4. Desprez P-Y, Roskelley C, Campisi J, Bissell MJ. (1993) Isolation of of functional cell lines from a mouse mammary epithelial cell strain: the importance of basement membrane and cell-cell interactions. *Mol. Cell. Differen.* **1**:99-110.
5. Desprez P-Y, Lin CQ, Thomasset N, Sympson CJ, Bissel MJ, Campisi J. (1998) A novel pathway for mammary epithelial cell invasion induced by the helix-loop-helix protein Id-1. *Mol. Cell. Biol.* **18**:4577-4588.
6. Cailleau R., Olive M., Cruciger QVJ. (1978) Long-term human breast carcinoma cell lines of metastatic origin: preliminary characterization. *In Vitro* **14**:911-915.
7. Dimri GP , Lee X, Basile G, Acosta M, Scott G, Roskelley C, Medrano EE, Linskens M, Rubelj I, Pereira-Smith O, Peacocke M, Campisi J. (1995) A Biomarker that Identifies Senescent Human Cells in Culture and in Aging Skin in vivo. *Proc. Natl. Acad. Sci. U.S.A.* **92**:9363-7.
8. Pendergrass WR, Lane MA, Bodkin NL, Hansen BC, Ingram DK, Roth GS, Yi L, Bin H, Wolf NS. (1999) Cellular proliferation potential during aging and caloric restriction in rhesus monkeys (Macaca mulatta). *J. Cell. Physiol.* **180**:123-30.
9. Mishima K, Handa JT, Aotaki-Keen A, Lutty GA, Morse LS, Hjelmeland LM. (1999) Senescence-associated beta-galactosidase histochemistry for the primate eye. *Ophtamol. Vis. Sci.* **40**:1590-3.
10. Campisi J. (2000) Cancer, aging and cellular senescence. *In Vivo* **14**:183-8.
11. Shelton DN, Chang, E, Whittier, PS, Choi D, Funk WD. (1999) Microarray analysis of replicative senescence. *Curr. Biol.* **9**:939-945.
12. Serrano M, Lin AW, McCurrach ME, Beach D, Lowe SW. (1997) Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. *Cell* **88**:593-602.
13. Chen QM, Bartholomew JC, Campisi J, Acosta M, Reagan JD, Ames BN. (1998) Molecular analysis of H2O2-induced senescent-like growth arrest in normal human fibroblasts: p53 and Rb control G1 arrest but not cell replication. *Biochem. J.* **332**:43-50.
14. Krtolica, A., S. Parrinello, S. Lockett, P.-Y. Desprez and J. Campisi. Senescent Fibroblasts Promote Epithelial Cell Growth and Tumorigenesis, Linking Cancer and Aging. *Proc. Natl. Acad. Sci. U.S.A.*, in press.

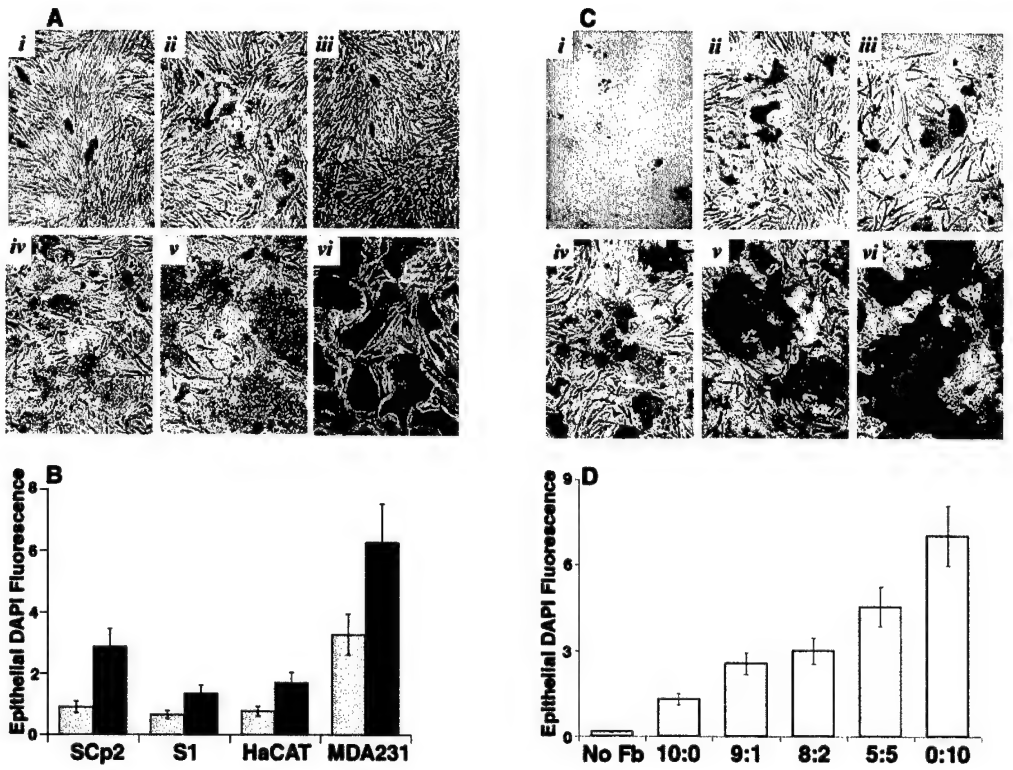


Figure 1

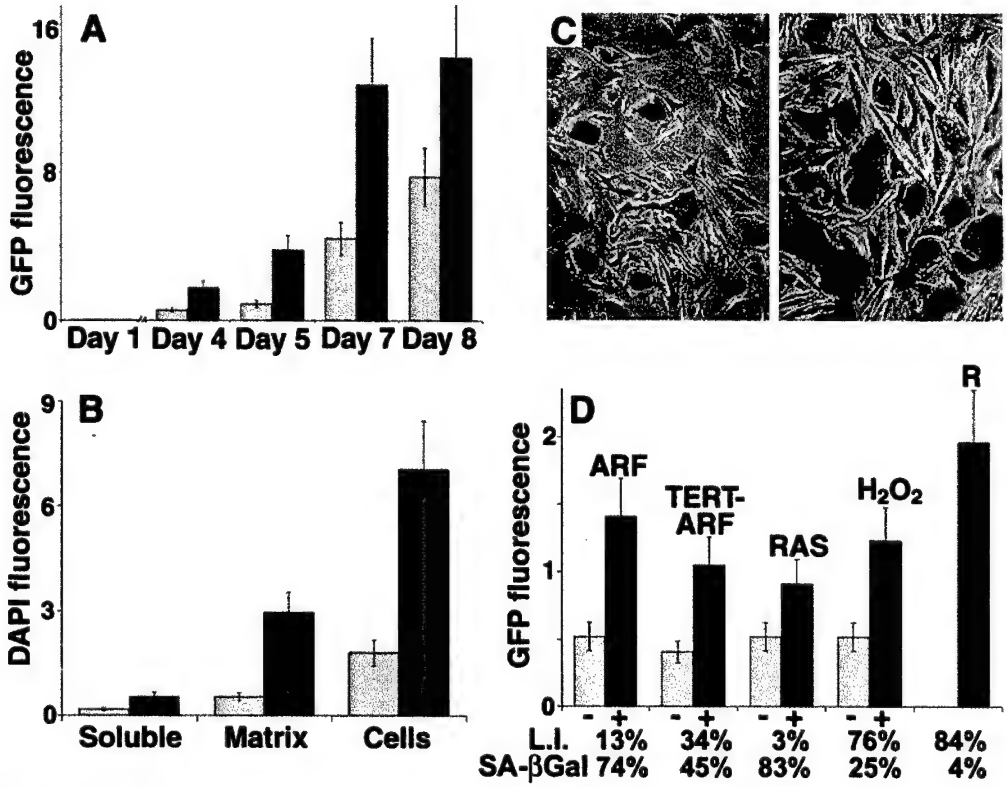


Figure 2

Appendices

List of Personnel

Ana Krtolica, P.I.

Biological Sciences, Cell Biology

Senescent Fibroblasts Promote Epithelial Cell Growth and Tumorigenesis: A Link between Cancer and Aging

Ana Krtolica[1], Simona Parrinello[1], Stephen Lockett[1†],

Pierre-Yves Desprez[2] and Judith Campisi[1]

[1] Life Sciences Division, Lawrence Berkeley National Laboratory, Berkeley, CA 94720

[2] Geraldine Brush Cancer Research Institute, California Pacific Medical Center, San Francisco, CA 94115

† Current Address: National Cancer Institute, SAIC-Frederick, Frederick, MD 21702

Corresponding author: Judith Campisi, Berkeley National Laboratory, 1 Cyclotron Rd., MS 84-171, Berkeley, CA 94720

Telephone: 510 486-4416; Fax: 510 486-4545; Email: JCAMPISI@LBL.GOV.

Text: **21 pgs**

Words in abstract: **168**

Characters: **36,038**

Mammalian cells can respond to damage or stress by entering a state of arrested growth and altered function termed cellular senescence. Several lines of evidence suggest that the senescence response suppresses tumorigenesis. Cellular senescence is also thought to contribute to aging, but the mechanism is not well understood. We show that senescent human fibroblasts stimulate premalignant and malignant, but not normal, epithelial cells to proliferate in culture and form tumors in mice. In culture, the growth stimulation was evident when senescent cells comprised only 10% of the fibroblast population, and was equally robust whether senescence was induced by replicative exhaustion, oncogenic RAS, $p14^{ARF}$ or hydrogen peroxide. Moreover, it was due at least in part to soluble and insoluble factors secreted by senescent cells. In mice, senescent, much more than presenescent, fibroblasts caused premalignant and malignant epithelial cells to form tumors. Our findings suggest that, although cellular senescence suppresses tumorigenesis early in life, it may promote cancer in aged organisms, suggesting it is an example of evolutionary antagonistic pleiotropy.

Multicellular organisms have evolved mechanisms to prevent the unregulated growth and malignant transformation of proliferating cells. One such mechanism is cellular senescence, which arrests proliferation – essentially irreversibly – in response to potentially oncogenic events. Cellular senescence appears to be a major barrier that cells must overcome to progress to full-blown malignancy (1-3).

Cellular senescence was first described as a process that limits the proliferation of cultured human fibroblasts (replicative senescence). Proliferating cells progressively lose telomeric DNA, and short telomeres, which are potentially oncogenic, elicit a senescence response. In addition, DNA damage, oncogene expression and supraphysiological

mitogenic signals cause cellular senescence. Cellular senescence is controlled by tumor suppressor genes, and appears to be a checkpoint that prevents the growth of cells at risk for neoplastic transformation (2,3). In this regard, cellular senescence is similar to apoptosis. However, whereas apoptosis kills and eliminates damaged or potential cancer cells, cellular senescence stably arrests their growth.

Cellular senescence is also thought to contribute to aging (4-7), although how it does so is poorly understood. In addition to arresting growth, senescent cells show changes in function (2,6,7). Because senescent cells accumulate with age (8-10), they may contribute to age-related declines in tissue function. If so, cellular senescence may be an example of antagonistic pleiotropy. Aging phenotypes are thought to result from the declining force of natural selection with age. Consequently, traits selected to maintain early life fitness can have unselected deleterious effects late in life, a phenomenon termed antagonistic pleiotropy (11). The senescence-induced growth arrest may suppress the development of cancer in young organisms. The functional changes, by contrast, may be unselected consequences of the growth arrest, and thus compromise tissue function as senescent cells accumulate.

Cellular senescence has been extensively studied in stromal fibroblasts from humans and mice. Upon senescence, such cells show striking changes in gene expression (7,12), some of which relate to the growth arrest and senescent morphology. Other changes, however, relate to fibroblast function. Senescent fibroblasts secrete growth factors, cytokines, extracellular matrix, and degradative enzymes (2,7,13), all of which can alter tissue microenvironments and affect nearby epithelial cells. Interestingly, this secretory phenotype resembles that of fibroblasts adjacent to some carcinomas, although senescent and tumor-associated fibroblasts differ in growth potential, morphology and other traits. Tumor-associated fibroblasts can stimulate epithelial tumorigenesis (14).

Because senescent cells can alter the tissue microenvironment, we and others proposed that senescent cells may contribute to the exponential rise in cancer that occurs with age (2,4,13,15). It is now clear that non-mutational events, such as telomere dysfunction or epigenetic changes in gene regulation or the stromal milieu, are important for the development of late life cancers (15). Changes in the stroma, which supports and maintains epithelial functions, may be particularly important in humans, where most age-related cancers arise from epithelial cells (15).

Here, we show that senescent human fibroblasts promote the proliferation and tumorigenesis of mutant epithelial cells. Our data suggest that cellular senescence is antagonistically pleiotropic, protecting from cancer early in life, but promote carcinogenesis in aged organisms.

MATERIALS AND METHODS

Cells. WI-38 (ATTC), 82-6 (J. Oshima, U. Washington), and 184 (M. Stampfer, P. Yaswen, LBNL) fibroblasts senesce after ~50, 40 and 25 doublings, respectively, and were cultured as described (8,16). Presenescent and senescent cultures generally contained >70% and <10% proliferating cells, and were <10% and >70% senescence-associated β-galactosidase (SA-βGal) positive, respectively (8,16). HaCAT (A. Paller, Northwestern U.), S1 (M. Bissell, LBNL), Ha(Pk) (L. Packer, U. California Berkeley), and MDA231 (R. Lupu, LBNL) cells were cultured as described (17-20). Keratinocytes (Clonetics) were cultured as directed by the supplier, and used 2-3 passages after receipt. 82-6 and WI-38 were immortalized with an hTERT-expressing retrovirus (21).

Co-cultures. Presenescent ($5x10^4$) and senescent ($1x10^5$) fibroblasts were allowed to attach to 6-well culture dishes overnight, and incubated in serum-free medium

for 1-3 d to generate lawns with similar cell numbers. Epithelial cells were incubated in growth factor-deficient medium for 2-3 d, plated ($2x10^4$/well) on fibroblast lawns, and maintained in growth factor-deficient medium for 8 d, unless noted otherwise. Growth factor-deficient medium contained: KBM, 1.8 mM $CaCl_2$, 5 μg/ml insulin, 0.5 μg/ml hydrocortisone for HaCAT and normal keratinocytes; DME/F12, 5 μg/ml insulin, 1.4 μM hydrocortisone, 5 μg/ml prolactin for SCp2 cells; DME/F12, 250 ng/ml insulin, 10 μg/ml transferrin, 2.6 ng/ml Na_2SeO_3, 1 ng/ml EGF, 0.1 nM estradiol, 1.4 μM hydrocortisone, 5 μg/ml prolactin for S1 cells; DME/F12, 5 μg/ml insulin for MDA231 cells. Cultures were fixed in 4% paraformaldehyde, and stained with 1% Rhodanile Blue (Sigma) or 1 μg/ml 4,6-diamidino-2-phenylindole (DAPI).

Soluble and matrix factors. Epithelial cells were seeded in upper chambers of Millicells-PCF (Millipore) containing presenescent or senescent lawns in the lower chambers, or onto matrices prepared by culturing fibroblasts in serum-free medium for 2-3 d, removing the cells with EDTA (Versene, GIBCO-BRL) at 37° C or 0.05% NP-40 at 23° C (with similar results), and washing with serum-free medium.

Quantification of epithelial cells. Fluorescent images from five random fields/well were captured at 400X and analyzed using a modification of SCIL_image 1.3 (TNO Inst. Appl. Physics, U. Amsterdam). The program was customized to recognize differences in DAPI fluorescence between epithelial (smaller, more intense) and fibroblast (larger, less intense) nuclei, and calculate epithelial fluorescence/field. We verified that epithelial DAPI signal is proportional to cell number by expressing enhanced green fluorescence protein (EGFP) using a retrovirus (LXSN) (22), and comparing epithelial DAPI and EGFP fluorescence in co-cultures. EGFP was expressed in >90% of cells.

Senescence induced by p14^{ARF}, RAS-Ha(V12) and H_2O_2. Fibroblasts infected with p14^{ARF} (22) or RAS-Ha(V12) retroviruses (23) were selected in puromycin (22), incubated without puromycin for 2-3 (p14^{ARF}) or 3-4 (RAS) d (22,23), and replated for co-culture as described above. Confluent presenescent fibroblasts were given 550 μM H_2O_2 in serum-containing medium for 2 h, left to recover overnight, then replated at subconfluence (24). After 5-7 d, cells developed a senescent phenotype and were replated for co-culture.

Tumorigenesis assays. Nude (*nu/nu*) mice (5 wk old) were injected (100 μl vol) subcutaneously into the dorsal flap with 1.5 x 10^6 HaCAT or Ha(Pk) cells, alone or with 1.5 x 10^6 fibroblasts, or nipple region with 1 x 10^6 SCp2 cells, alone or with 1 x 10^6 fibroblasts, or 2.5 x 10^5 MDA231 alone or with 1 x 10^6 fibroblasts. We measured 3 maximum diameters at *x*, *y* and *z* axes to derive tumor volume (size). Tumors were fixed in buffered formalin, or embedded in Tissue-Tek (Sakura) and snap frozen, with or without prior fixation in 4% paraformaldehyde and passage through a sucrose gradient. Fixed frozen sections were blocked in 1% bovine serum albumin, and stained with pan-cytokeratin antibody (1:100; Dako) for 1 h and FITC-secondary antibody for 45 min.

RESULTS

Senescent fibroblasts stimulate preneoplastic epithelial cell growth. We preincubated presenescent and replicatively senescent human fibroblasts in serum-free medium to arrest the presenescent cells, and used equal numbers of non-dividing presenescent and senescent cells to produce 50-80% confluent lawns onto which epithelial cells were seeded. We used four epithelial cell lines. HaCAT human epidermal keratinocytes (17), S1 human mammary epithelial cells (18), and SCp2 mouse

mammary epithelial cells (19) are immortal, harboring p53 mutations, but do not form tumors in immunocompromised mice (17,18,25; see Fig. 4). Thus, they are preneoplastic, having acquired only some mutations that predispose to malignancy. In addition, we used MDA231, an aggressive human breast cancer cell line (20). We also used normal human keratinocytes from adult or neonatal donors. Like the fibroblasts, these normal strains have a finite replicative capacity and no known mutations that predispose to malignancy. We preincubated epithelial cells in growth-factor-deficient medium before seeding them in this medium onto fibroblast lawns.

We first evaluated preneoplastic and neoplastic epithelial cells co-cultured with fibroblasts using Rhodanile Blue, which preferentially stains epithelial colonies (Fig. 1A). Senescent fibroblasts, much more than presenescent fibroblasts, stimulated the growth of all four cell lines. To quantify this, we stained the co-cultures with DAPI, a fluorescent DNA dye, and quantified the smaller, more intensely stained epithelial nuclei by image analysis (Fig. 1B). Alternatively, we expressed enhanced green fluorescent protein (EGFP) in the epithelial cells, and measured EGFP fluorescence (see Fig. 3A). Compared to presenescent fibroblasts, senescent fibroblasts stimulated HaCAT, S1 and MDA231 cells 2- to 4-fold, and SCp2 cells 3- to 7-fold (Fig. 1B). Preliminary data suggest that senescent fibroblasts also stimulate the growth of sarcoma cells (HT1080 fibrosarcoma, SOAS-2 osteosarcoma), but to a lesser extent (50-80% stimulation).

The growth stimulation was not due to epithelial cells attaching better to senescent fibroblasts. One day after seeding, DAPI fluorescence showed that epithelial cells attached equally well to presenescent and senescent lawns (not shown). This differential growth stimulation was seen with three human fibroblast strains: WI-38 (fetal lung; Fig. 1), 82-6 (adult skin; Fig. 3), and 184 (adult breast; not shown).

Cells expressing a senescence marker (8) accumulate with age (8,9,10), but remain relatively rare, even in old tissues. To determine whether senescent fibroblasts stimulate preneoplastic epithelial cells even when abundant presenescent fibroblasts are present, we cultured SCp2 cells alone or on lawns containing varying fractions of senescent fibroblasts, and stained with Rhodanile (Fig. 1C) or DAPI (Fig. 1D) 8 d later. SCp2 proliferation was minimal in the absence of fibroblasts (Fig. 1C, panel *i*; 1D, No Fb). Presenescent fibroblasts stimulated growth 5-8 fold (Fig. 1C, panel *ii*; 1D, 10:0), which could be caused by presenescent fibroblasts *per se*, or by the 10-30% senescent cells always present in presenescent cultures. Whatever the case, increasing proportions of senescent fibroblasts progressively stimulated additional SCp2 growth, even when senescent cultures were only 10% of the fibroblast population (Figs. 1C-D). This experiment also suggests that presenescent fibroblasts do not inhibit the growth of preneoplastic epithelial cells, but, rather, that senescent cells facilitate their growth.

Senescent fibroblasts do not stimulate normal epithelial cells. In striking contrast to preneoplastic and neoplastic epithelial cells, genetically normal keratinocytes grew equally well on presenescent and senescent fibroblasts. After 8 d in co-culture, there was no statistical difference between growth on presenescent and senescent lawns. This was true for neonatal and adult human keratinocytes (Fig. 2), and human mammary epithelial cells (not shown). Thus, although senescent fibroblasts stimulated preneoplastic and malignant epithelial cells, they did not differentially stimulate normal epithelial cells.

Kinetics. To follow the kinetics of the growth stimulation, we seeded EGFP-expressing HaCAT cells onto presenescent or senescent fibroblast lawns, and monitored EGFP fluorescence with time (Fig. 3A). Growth on senescent fibroblasts surpassed that

on presenescent fibroblasts within 4 d (Fig. 3A), and continued to do so until the epithelial cells reached confluence (after 9-10 d; not shown). Unless noted otherwise, subsequent experiments were terminated within 8 d.

Contribution of secreted factors. Senescent fibroblasts might stimulate epithelial cell growth by direct cell-cell interaction, or by secreting diffusible factors or an insoluble extracellular matrix.

To test these possibilities, we cultured cells in two-chamber dishes. These separated fibroblast lawns from the epithelial cells by a porous membrane (0.4 μm pore size), preventing direct contact but permitting exchange of soluble diffusible factors. Soluble factors secreted by senescent fibroblasts were 2- to 3-fold more potent in stimulating HaCAT (not shown) and SCp2 (Fig. 3B) growth than those secreted by presenescent fibroblasts. Overall, however, senescent fibroblast-derived soluble factors were 10-fold less potent than direct cell contact (Fig. 3B, Soluble vs Cells, black bars), even though epithelial cells attached equally well to the membrane and fibroblasts (not shown).

To determine the contribution of secreted matrices, we allowed fibroblasts in serum-free medium to deposit extracellular matrix onto culture dishes for 2-3 days. We then removed the cells by calcium chelation or mild detergent. Immunocytochemistry showed that fibroblast-depleted dishes contained abundant fibronectin (not shown), suggesting that at least this matrix component survived cell removal. We plated SCp2 cells onto the matrices, and quantified cell number by DAPI fluorescence. Matrix produced by senescent fibroblasts was 3- to 4-fold more stimulatory than matrix

produced by presenescent fibroblasts (Fig. 3B). This difference was not due to differences in epithelial cell attachment (not shown).

Together, these results indicate that about 10% of the growth stimulation caused by senescent fibroblasts was due to secreted soluble factors, while 40% was due to secreted extracellular matrix. These are minimal estimates because cells may experience higher levels of soluble factors in direct co-culture, and/or matrix components may be lost or inactivated during cell removal. Thus, at least 50% of the growth stimulation was attributable to the secretory phenotype of senescent fibroblasts.

Growth stimulation is independent of the senescence inducer. Overexpression of certain oncogenes or tumor suppressor genes or DNA damage can induce a phenotype that closely resembles replicative senescence (2,12). We therefore asked to what extent the ability to stimulate preneoplastic epithelial cells depended on replicative exhaustion.

First, we induced senescence by overexpressing p14*ARF*, a tumor suppressor that mediates a senescence response (22). We seeded HaCAT cells onto lawns of presenescent or p14*ARF*-arrested fibroblasts and assessed HaCAT number by EGFP fluorescence. p14*ARF*-arrested fibroblasts stimulated HaCAT cell growth 2- to 3-fold relative to presenescent fibroblasts (Fig. 3C, D).

We next tested 82-6 fibroblasts that we immortalized with hTERT (21), the catalytic subunit of human telomerase (26,27). The immortal cells had completed >80 doublings, or twice the replicative life span of unmodified control cells. HaCAT cells grew equally well on immortal and presenescent control fibroblasts [Fig. 3D; (–, ARF) vs (–, TERT-ARF)]. Thus, cell division *per se* was not responsible for the stimulation

caused by replicatively senescent fibroblasts. In response to p14^{ARF}, the immortal fibroblasts arrested growth with a senescent morphology (not shown), whereupon they stimulated HaCAT growth 2- to 3-fold [Fig. 3D, (+, ARF) vs (+, TERT-ARF)].

We also induced senescence by expressing oncogenic RAS [RAS-Ha(V12)] (23), and seeded HaCAT cells onto presenescent or RAS-arrested fibroblasts. RAS-arrested fibroblasts stimulated HaCAT growth 1.5-fold compared to presenescent fibroblasts (Fig. 3D, RAS). This stimulation may be an underestimate because we analyzed the co-cultures after only 5 d, owing to poor survival of RAS-expressing cells after >5 d in growth factor-deficient medium.

Finally, we treated fibroblasts with a sublethal dose (550 μM) of H_2O_2, allowed them to develop a senescent phenotype (24), and seeded HaCAT cells onto untreated or H_2O_2-treated fibroblasts. HaCAT cells proliferated 2- to 3-fold more readily on the H_2O_2-arrested fibroblasts (Fig. 3D, H_2O_2).

Thus, fibroblasts induced to senesce by several means stimulated the proliferation of preneoplastic epithelial cells. This stimulation was 50-70% of that caused by replicatively senescent fibroblasts (Fig. 3D, R), indicating that, at least qualitatively, the growth stimulation was independent of the senescence inducer.

Senescent fibroblasts stimulate tumorigenesis. To test the idea that senescent cells create a microenvironment that promotes the growth of potentially or frankly neoplastic cells *in vivo*, we injected epithelial cells, alone or with fibroblasts, into immunocompromised (*nu/nu*) mice (Fig. 4).

HaCAT cells alone did not form tumors after 40 (Fig. 4A, panel *i*) or 180 (not shown) d, as reported (17). When injected with an equal number of presenescent fibroblasts, 3/20 animals developed small tumors (<20 mm^3); one regressed and two were excised for histology (Fig. 4A, panel *ii*). Most of the animals (17/20), however, were tumor-free after >100 d. By contrast, when HaCAT were injected with senescent fibroblasts, 7/15 animals developed tumors that averaged twice the size of those formed by presenescent fibroblasts (P=<0.01) (Fig. 4A, panel *iii*). Most of the tumors began to regress before excision, suggesting they were not malignant. This was not the case with SCp2 cells. SCp2 cells also did not form tumors (Fig. 4B, panel *i*), as reported (25). Moreover, when injected with presenescent fibroblasts, they did not form tumors after 120 (Fig. 4B, panel *ii*) or 160 (not shown) d. However, when injected with senescent fibroblasts, they formed very large (400-2000 mm^3) tumors in 4/6 animals (Fig. 4B, panel *iii*) with a latency of >80 d. These tumors would have killed the animals if not excised. Thus, senescent fibroblasts stimulated hyperproliferation and neoplastic progression, respectively, of HaCAT and SCp2 cells *in vivo*.

To determine effects on malignant epithelial cells, we used Ha(Pk), a weakly tumorigenic HaCAT derivative (unpublished), and MDA231, an aggressive human breast cancer cell line (20). Ha(Pk) cells alone did not form tumors after 40 d (Fig. 4C, panel *i*), but did form tumors in 4/5 animals after 90 d (not shown). Presenescent fibroblasts accelerated tumorigenesis: within 25 d, 3/5 mice developed small (30-50 mm^3) tumors that grew slowly over the next 2 wks (Fig. 4C, panel *ii*). Senescent fibroblasts accelerated tumorigenesis further: 4/5 mice developed tumors, two of which appeared in <10 d and grew rapidly (>100 mm^3) (Fig. 4C, panel *iii*). More striking results were obtained with MDA231 cells. Small numbers (2 x 10^5) of MDA231 cells produced

tumors that reached 300-400 mm^3 in 2/5 mice in 45 d (Fig. 4D, panel *i*). hTERT-immortalized fibroblasts slightly accelerated tumorigenesis, causing tumors that reached 100-500 mm^3 in 4/5 mice (Fig. 4D, panel *ii*). Senescent fibroblasts greatly accelerated tumorigenesis. Four of five mice developed tumors that reached 300-1600 mm^3 (Fig. 4D, panel *iii*). Thus, at least for HaCAT, Ha(Pk) and MDA231, presenescent or hTERT-immortalized fibroblasts did not inhibit tumorigenesis. More importantly, senescent fibroblasts strongly stimulated or facilitated tumorigenesis.

Characteristics of tumors. We used EGFP-expressing HaCAT cells to confirm that tumors originated from the injected epithelial cells (Fig. 5A, B). HaCAT tumors typically were firm nodules. In the presenescent group, tumors were often keratinized with sharply demarcated margins (Fig. 5C). By contrast, the senescent group frequently showed poorly defined margins with little keratinization (Fig. 5D). Tumors formed by Ha(Pk) cells were high-grade, dysplastic epidermoid cysts, with 5- to 10-fold more mitoses in the senescent group (not shown).

SCp2 tumors, which formed only in the presence of senescent fibroblasts (Fig. 4B), were highly anaplastic, with frequent mitoses and features of malignant sarcoma (Fig. 5F). A striking feature was the loss of cytokeratin (Fig. 5E, F), in contrast to abundant cytokeratin in the starting SCp2 cells (19,25) and host mammary epithelium (Fig. 5E, F). Senescent fibroblasts may induce an epithelial to mesenchymal transition in these cells, or selectively stimulate variants that lost epithelial characteristics.

MDA231 cells formed poorly differentiated, aggressive tumors with characteristics of invasive ductal carcinoma, as reported (20). Aside from size, there were no discernible differences among tumors formed by presenescent and senescent

fibroblasts (Fig. 5G, H). Thus, senescent fibroblasts accelerated the growth, but did not influence the phenotype, of these tumors.

DISCUSSION

Several lines of evidence indicate that cellular senescence suppresses tumorigenesis *in vivo* (1-3). First, many tumors contain cells that have partially or completely overcome senescence. Second, several oncogenes act at least partly by disabling the senescence checkpoint. Third, the senescence response requires p53 and pRB, the two most commonly lost tumor suppressors in malignant tumors. Finally, germline inactivation of the p53 or pRB pathways results in senescence-defective cells, and cancer-prone organisms. Despite species differences in whether and how cells respond to specific senescence-inducing stimuli, cellular senescence very likely protects mammals from cancer, at least early in life.

We found that senescent human fibroblasts stimulated hyperproliferation and progression of preneoplastic epithelial cells, and accelerated tumorigenesis by neoplastic epithelial cells. These results may seem at odds with the tumor suppression function of cellular senescence. They are, however, consistent with the evolutionary theory of antagonistic pleiotropy (11), which predicts that some genes, selected to enhance the fitness of young organisms, can have unselected deleterious effects in aged organisms. Our findings suggest that cellular senescence, despite protecting from cancer in young adults, may promote cancer progression in aged organisms. We speculate that the growth arrest was selected to ensure that damaged, mutant, or inappropriately stimulated cells – cells at risk for neoplastic transformation – do not proliferate. By contrast, the functional changes may be unselected consequences of the growth arrest, having little impact on

young organisms where senescent cells are rare (8-10). However, as damage, telomere attrition, or errors cause senescent cells to accumulate with age, their influence, particularly their secretory phenotype, may become significant and deleterious.

Senescent fibroblasts had little impact on the growth of normal epithelial cells, although they can disrupt tissue architecture and function (28). However, they clearly stimulated preneoplastic and neoplastic cell growth, largely due to the secretion of both soluble and insoluble factors. Our results suggest that senescent cells produce multiple factors, which act together, to stimulate epithelial cells with oncogenic mutations.

Somatic mutations increase with age (29,30), and some are potentially oncogenic. For example, loss of heterozygosity and mutations in p53 and RAS-Ha accumulate in normal adult tissue (31-33). We suggest that, with age, there is an increasing probability that senescent cells and cells with oncogenic mutations occur in close proximity. Senescent cells, then, may create a microenvironment that facilitates the growth and progression of the mutant cells. Although tumors in older organisms tend to be more indolent, neoplastic cells were more likely to form tumors in older animals (34).

The senescent microenvironment may synergize with multiple factors to contribute to late life cancers (15). In addition to mutations, these include telomere dysfunction, hormonal and immune status, and angiogenic potential of the tissue. It is still not clear why most late life cancers are epithelial. Sarcomas may be less prone to stimulation by senescent fibroblasts, but other factors -- a greater need for mutations or a young hormonal or angiogenic milieu -- may explain the relative paucity of sarcomas.

In summary, our results suggest a new link between cancer and aging, and a plausible mechanism by which genetic (oncogenic mutations) and epigenetic (accumulation of senescent cells) events synergize to generate the exponential rise in cancer that occurs with aging.

REFERENCES

1. Yeager, T. R., et al. (1998) *Genes Dev.* **12**, 163-174.
2. Campisi, J. (2000) *In Vivo* **14**, 183-188.
3. Reddel, R. R. (2000) *Carcinogenesis* **21**: 477-484.
4. Campisi, J. (1997) *J. Am. Geriatrics Soc.* **45**, 482-488.
5. Smith, J. R., & Smith, O. M. (1996) *Science* **273**, 63-67.
6. Campisi, J. (1996) *Cell* **84**, 497-500.
7. Campisi J., Dimri G. P., & Hara, E. (1996) in *Handbook of the Biology of Aging*, Eds. Schneider E. & Rowe J., (Academic Press, New York), pp. 121-149.
8. Dimri, G. P., et al. (1995) *Proc. Natl. Acad. Sci. U.S.A.* **92**, 9363-9367.
9. Pendergrass, W. R., Lane, M. A., Bodkin, N. L., Hansen, B. C., Ingram, D. K., Roth, G. S., Yi, L., Bin, H., Wolf, N. S. (1999) *J. Cell. Physiol.* **180**, 123-130.
10. Mishima, K., Handa, J. T., Aotaki-Keen, A., Lutty, G. A., Morse, L. S., & Hjelmeland, L. M. (1999) *Ophtamol. Vis. Sci.* **40**, 1590-1593.
11. Rose, M. R. (1991) *The Evolutionary Biology of Aging* (Oxford Univ. Press, NY).
12. Shelton, D. N., Chang, E., Whittier, P. S., Choi, D., & Funk, W. D. (1999) *Curr. Biol.* **9**, 939-945.
13. Rinehart, C. A., & Torti, V. R. (1997) *Molec. Carcinog.* **18**, 187-192.
14. Olumi, A. F., Grossfeld, G. D., Hayward, S. W., Carroll, P. R., Tlsty, T. D., & Cunha, G. R. (1999) *Cancer Res.* **59**, 5002-5011.

15. DePinho, R. (2000) *Nature* **408**, 248-254.

16. Seshadri T, Campisi J. (1990) *Science* **247**, 205-209.

17. Skobe, M., & Fusenig, N. E. (1998) *Proc. Natl. Acad. Sci. U.S.A.* **95**, 1050-1055.

18. Briand, P., Petersen, O. W., & van Deurs, B. (1987) *In Vitro Cell Dev. Biol.* **23**, 181-188.

19. Desprez, P. Y., Roskelley, C., Campisi, J., & Bissell, M. J. (1993) *Mol. Cell. Differen.* **1**, 99-110.

20. Cailleau, R., Olive, M., & Cruciger, Q. V. J. (1978) *In Vitro* **14**, 911-915.

21. Kim S. H., Kaminker P., & Campisi J. (1999) *Nature Genetics* **23**, 405-412.

22. Dimri, G. P., Itahana, K., Acosta, M., & Campisi, J. (2000) *Mol. Cell. Bio.* **20**, 273-285.

23. Serrano, M., Lee, A. W., McCurrach, M. E., Beach, D., & Lowe, S. W. (1997) *Cell* **88**, 593-602.

24. Chen, Q. M., Bartholomew, J. C., Campisi, J., Acosta, M., Reagan, J. D., & Ames, B. N. (1998) *Biochem. J.* **332**, 43-50.

25. Desprez, P.-Y., Lin, C. Q., Thomasset, N., Sympson, C. J. Bissell, M. J., & Campisi, J. (1998) *Mol. Cell. Biol.* **18**, 4577-4588.

26. Bodnar, A. G., Ouellette, M., Frokis, M., Holt, S. E., Chiu, C.-P., Morin, G. B., Harley, C.B., Shay, J. W., Lichtsteiner, S., & Wright, W. E. (1998) *Science* **279**, 349-352.

27. Meyerson, M., Counter, C. M., Eaton, E. L., Ellisen, L. W., Steiner, P., Caddle, S. D., Ziaugra, L., Beijersbergen, R. L., Davidoff, M., Liu, Q., Bacchetti, S., Haber, D. A., & Weinberg, R. A. (1997) *Cell* **90**, 785-795.

28. Funk, W. D., Wang, C. K., Shelton, D. N., Harley, C. B., Pagon, G. D., & Hoeffler, W. K. (2000) *Exp. Cell Res.* **258**: 270-278.

29. Dollé, M. E., Giese, H., Hopkins, C. L., Martus, H. J., Hausdorff, J. M., & Vijg, J. (1997) *Nat. Genet*. **17**, 431-434.

30. Dolle, M. E., Snyder, W. K., Gossen, J. A., Lohman, P. H., & Vijg, J. (2000) *Proc. Natl Acad. Sci. USA* **97**, 8403-8408.

31. Deng, G., Lu, Y., Zlotnikov, G., Thor, A. D., & Smith, H. S. (1996) *Science* **274**, 2057-2059.

32. Jonason, A. S., Kunala, S., Price, G. T., Restifo, R. J., Spinelli, H. M., Persing, J. A., Leffell, D. J., Tarone, R. E., & Brash, D. E. (1996) *Proc. Natl. Acad. Sci. USA* **93**, 14025-14029.

33. Cha, R. S., Thilly, W. G., & Zarbl, H. (1994) *Proc. Natl. Acad. Sci. USA* **91**, 3749-3753.

34. McCullough, K. D., Coleman, W. B., Smith, G. J., & Grisham, J. W. (1997) *Cancer Res*. **57**, 1807-1813.

Acknowledgements: We thank R. Weinberg for the hTERT cDNA, S. Lowe for the RAS-Ha(V12) vector, D. Yip for preliminary experiments, W. Chou for help with computer analyses, the S. Fisher laboratory for help with tumor analyses, V. Band and R. Lupu for advice on tumor studies, L. Frappart for pathological evaluation of the tumors, and S. Hayward, G. Cunha and T. Tlsty for discussions. Supported by DOD fellowship DAMD17-98-1-8063 (A. K.), and NIH grants AG09909 (J. C.) and CA82548 (P. D.).

FIGURE LEGENDS

Fig. 1. Effects on preneoplastic and malignant epithelial cells. Epithelial cells were plated on WI-38 fibroblast lawns, and cultured in growth factor-deficient medium for 8 d.

A. S1 (*i*, *iv*), SCp2 (*ii*, *v*) and HaCAT (*iii*, *vi*) cells, cultured on presenescent (*i-iii*) or senescent (*iv-vi*) lawns, stained with Rhodanile (240X).

B. Co-cultures described in (A), and co-culture with MDA231 cells, stained with DAPI. Epithelial nuclei were quantified as described in Methods. The results (in arbitrary units) shown are from 1 of 3-5 experiments. Error bars = standard error of the means (SEM) of duplicate or triplicate wells. Gray bars, presenescent lawns; black bars, senescent lawns.

C. SCp2 cells cultured without fibroblasts (*i*), or with presenescent and senescent cultures at ratios 10:0 (*ii*), 9:1 (*iii*), 8:2 (*iv*), 5:5 (*v*), and 0:10 (*vi*), stained with Rhodanile (240X).

D. SCp2 cells cultured without fibroblasts (No Fb) or with ratios of fibroblasts described in (C), quantified by DAPI fluorescence. Error bars = SEM from triplicate wells from 1 of 2 experiments.

Fig. 2. Effects on normal epithelial cells. Normal adult (Adult) or neonatal (Neonatal) human keratinocytes were seeded onto WI-38 lawns, cultured for 8 d, and quantified by DAPI fluorescence. Error bars = SEM from duplicate wells from 1 of 2 experiments. Gray bars, presenescent lawns; black bars, senescent lawns.

Fig. 3. Characteristics of the growth stimulation.

A. EGFP-expressing HaCAT cells, seeded onto presenescent or senescent WI-38 lawns. EGFP fluorescence (in arbitrary units) was measured after 1-8 d, as indicated.

Error bars = SEM from duplicate wells from 1 of 2 experiments. Gray bars, presenescent lawns; black bars, senescent lawns.

B. SCp2 cells, seeded onto WI-38 fibroblasts (Cells), plated in the upper chambers of Millicells containing fibroblasts in the lower chambers (Soluble), or plated onto matrices deposited by fibroblasts (Matrix), as described in Methods. Cell number was assessed after 8 d by DAPI fluorescence. Error bars = SEM from duplicate wells from 1 of 2 experiments. Gray bars, presenescent fibroblasts; black bars, senescent fibroblasts.

C. HaCAT cells, seeded onto lawns of control (left) or p14*ARF*-expressing (right) presenescent 82-6 fibroblasts, stained 8 d later with Rhodanile (250X).

D. HaCAT cells, quantified by EGFP fluorescence after co-culture with control (–) or p14*ARF*-expressing (+) 82-6 fibroblasts (ARF); control (–) or p14*ARF*-expressing (+) hTERT-immortalized 82-6 cells (TERT-ARF); control (–) or RAS-Ha/V12-expressing (+) WI-38 cells (RAS); 82-6 cells untreated (–) or treated (+) with H_2O_2; or replicatively senescent WI-38 cells (R). Growth was assessed after 8 d, except for RAS panels, where growth was assessed after 5 d. Error bars = SEM from duplicate wells from 1 of 2 experiments. Gray bars, presenescent lawns; black bars, senescent lawns.

Fig. 4. Tumor growth stimulated by fibroblasts. Nude mice were injected with epithelial cells alone (Control) or presenescent (Presn), senescent (Sen), or hTERT-immortalized (Telom) fibroblasts. At the indicated intervals (Days), tumor size was measured as described in Methods. The number of animals per group (n=) is indicated. The last point on each line indicates when tumors were excised for histology.

A. HaCAT cells (1.5×10^6) alone or with 1.5×10^6 WI-38 fibroblasts. Shown are results from 2 experiments. The incidence of tumors >10 mm^3, and average tumor size, were greater in the senescent group ($p<0.05$).

B. SCp2 cells (1 x 10^6) alone or with 1 x 10^6 WI-38 cells. Tumor incidence was greater in the senescent group ($p<0.05$).

C. Ha(Pk) cells (1.5 x 10^6) alone or with 1.5 x 10^6 WI-38 cells.

D. MDA231 cells (2.5 x 10^5) alone or with 1 x 10^6 WI-38 cells. Tumor size was greater in the senescent group ($p<0.01$).

Fig. 5. Characterization of tumors induced by fibroblasts.

A. Fluorescence image of a frozen section of a tumor formed by EGFP-expressing HaCAT cells (80X). Tumors formed in the presence of presenescent or senescent fibroblasts were similarly fluorescent.

B. Section shown in (A), counterstained with DAPI (80X).

C. HaCAT tumor formed in the presence of presenescent WI-38 cells (fixed section, stained by hematoxylin/eosin) (160X). Arrow shows a well demarcated tumor margin.

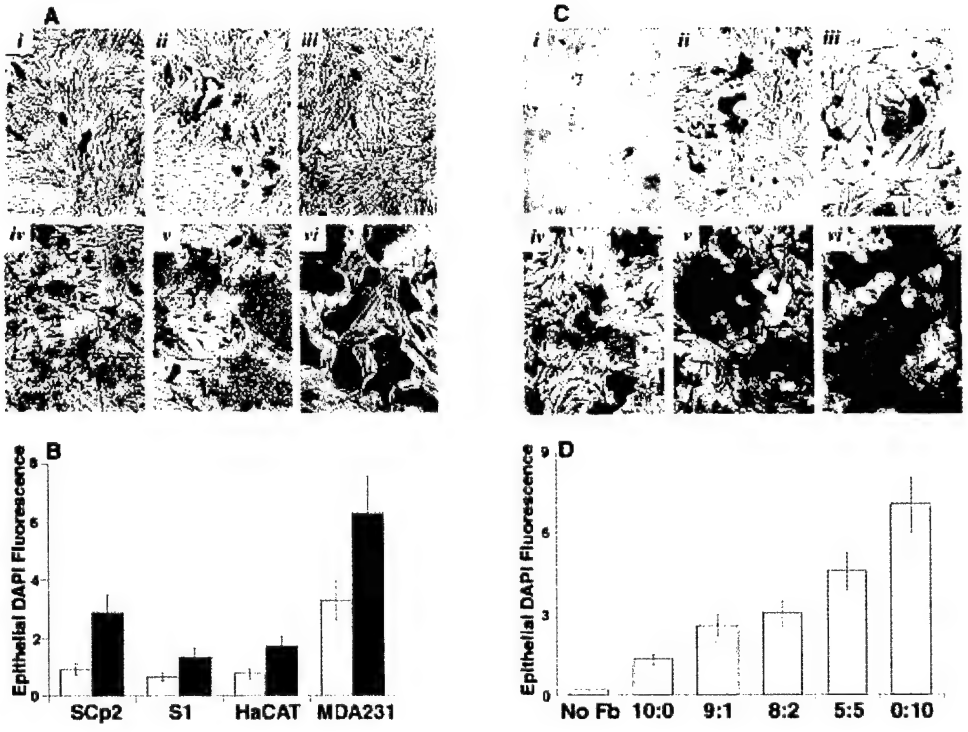
D. HaCAT tumor formed in the presence of senescent WI-38 cells (fixed section, stained by hematoxylin/eosin) (160X). Arrow shows a poorly demarcated tumor margin.

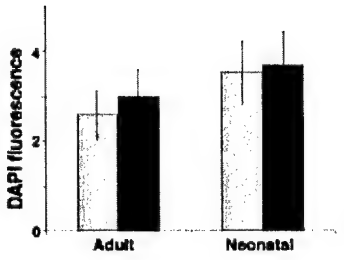
E. Fluorescence image of a frozen section of a SCp2 tumor formed by senescent WI-38 cells, stained for cytokeratin (160X). Solid arrow shows positive staining in the host mammary duct; open arrow shows lack of staining in surrounding tumor cells.

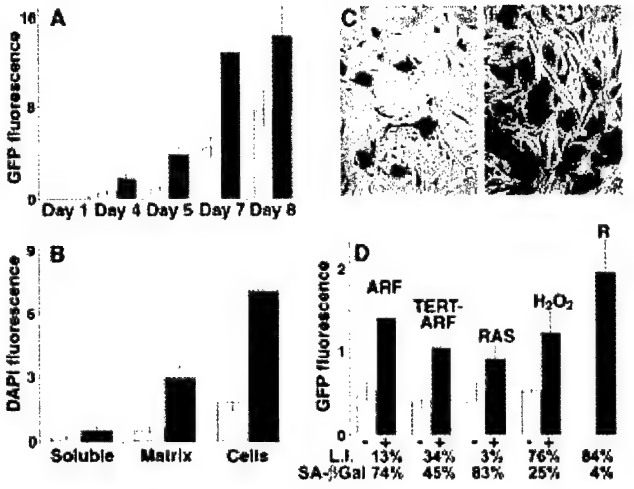
F. Frozen section adjacent to that shown in (E), stained by hematoxylin/eosin (160X). Solid arrow, host ductal epithelial cells; open arrow, surrounding anaplastic tumor cells.

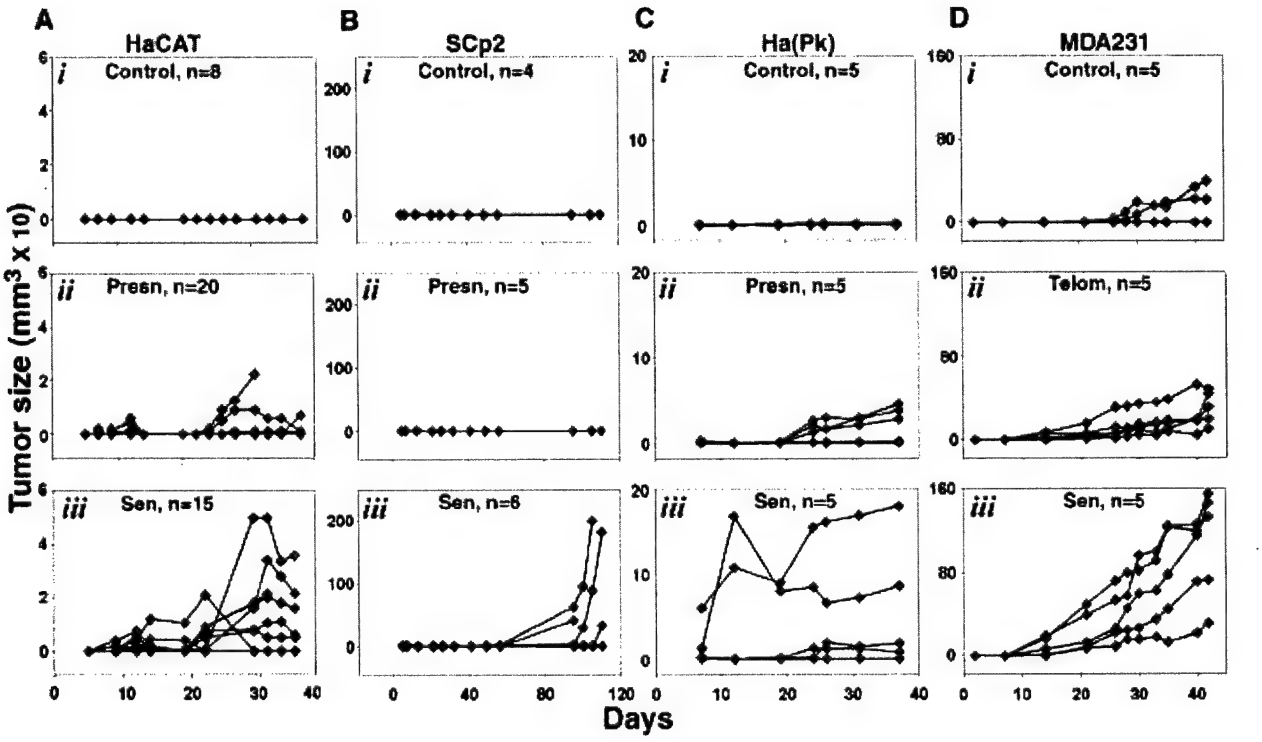
G. Paraffin-embedded section of a MDA231 tumor formed in the presence of presenescent WI-38 cells, stained with hematoxylin/eosin (40X).

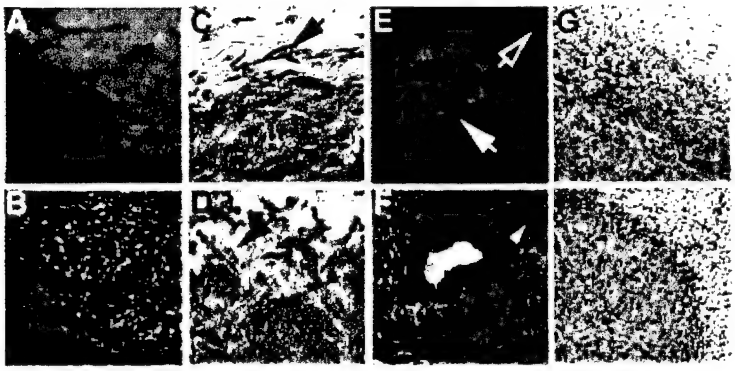
H. Paraffin-embedded section of a MDA231 tumor formed in the presence of senescent WI-38 cells, stained with hematoxylin/eosin (40X).


A
i
ii
iii
iv
v
vi
C
i
ii
iii
iv
v
vi
B
Epithelial DAPI Fluorescence
0
2
4
6
8
SCp2
S1
HaCAT
MDA231
D
Epithelial DAPI Fluorescence
0
3
6
9
No Fb
10:0
9:1
8:2
5:5
0:10


DAPI fluorescence
4
2
0
Adult
Neonatal


A
GFP fluorescence
16
8
0
Day 1 Day 4 Day 5 Day 7 Day 8
C
B
DAPI fluorescence
9
6
3
0
Soluble
Matrix
Cells
D
GFP fluorescence
2
1
0
ARF
TERT-ARF
RAS
H2O2
R
- + - + - + - +
L.I. 13% 34% 3% 76% 84%
SA-βGal 74% 45% 83% 25% 4%


A
HaCAT
i
Control, n=8
ii
Presn, n=20
iii
Sen, n=15
B
SCp2
i
Control, n=4
ii
Presn, n=5
iii
Sen, n=6
C
Ha(Pk)
i
Control, n=5
ii
Presn, n=5
iii
Sen, n=5
D
MDA231
i
Control, n=5
ii
Telom, n=5
iii
Sen, n=5
Tumor size (mm3 X 10)
Days


A
C
E
G
B
D
F

Ana Krtolica

Work:
Dept. of Cell and Molecular Biology
Life Sciences Division, M.S. 84-171
Lawrence Berkeley National Laboratory
Berkeley, CA 94720
E-mail: AKrtolica@lbl.gov
Tel.:(510) 486-4417
Fax: (510) 486-4545

Home:
1600 Clement St. Apt.303
San Francisco, CA 94121
Tel.: (415) 831-5897
E-mail: akrtolica@att.net

Research and Professional Experience

Post-Doctoral Research ***1997-present***

Supervisor Judith Campisi

I am currently investigating the role of aging stroma on development of cancer using breast and skin cancer models. To this end, I have established both 2-D and 3-D in vitro coculture systems, with and without direct cell-cell contact, to study the effects of stromal cells on epithelial cell growth, invasiveness and differentiation. I have confirmed the results obtained in these in vitro models using nude mouse tumorigenic assays. Additionally, I am using these models to investigate specific genetic alterations that sensitize mammary epithelial cells to the pro-carcinogenic microenvironment created by senescent fibroblasts.

Ph.D. Research ***1992-1997***

"The Effect of Hypoxia on Two Major Cancer Cell Characteristics – Uncontrolled Proliferation and Invasiveness."
Thesis Advisor John W. Ludlow

The goal of my Ph.D. thesis project was to elucidate the mechanism(s) involved in hypoxia induced cell cycle arrest, in particular the function of pRB activation (through accumulation in its underphosphorylated form) and the role of cyclin-cdk complexes and phosphatase activity.

Research Skills

Include: Extensive experience with mammalian cell and tissue culture, including co-culture, hypoxic chamber and 3-D systems using human and mouse primary (fibroblasts, keratinocytes, mammary epithelial, endothelial) and immortalized cells, flow cytometry and cell sorting, invasion assays, confocal microscopy, immunoprecipitation, substrate gels, enzymatic activity assays, immunochemistry, autohistoradiography, mouse tumorigenicity assays, Western blotting, Northern and Southern blotting and hybridization, PCR, mammalian cell transfection and infection, bacterial and yeast culture and manipulation, yeast two-hybrid system, subclonning techniques, DNA and RNA isolation, affinity chromatography, gel filtration.

In collaboration with Dr. Stephen Lockett, head of the Bioimaging and Microscopy laboratory in the Life Sciences Division of LBNL, I have developed a method for epithelial cell quantification in 2D coculture systems.

Publications

1. Krtolica, A. and J.W. Ludlow. "*Hypoxia Arrests Ovarian Carcinoma Cell Cycle Progression, but Invasion is Unaffected.*" Cancer Research 56:1168-1173, 1996.

   We developed an *in vitro* model system to study the molecular mechanisms involved in the proliferation and invasion of human ovarian carcinoma cells under hypoxia. We show that hypoxia induces reversible G0/G1 cell cycle arrest that is concomitant with accumulation of dephosphorylated pRB and decrease in cyclin A abundance. Ovarian carcinoma cell lines under hypoxic conditions retained their invasion potential and MMP2 expression, suggesting that local metastasis may occur even in low oxygen.

2. Khan, S., E. Matysiak-Zablocki, R. Ball, A. Krtolica, G. Hawkins, M. Ghahremani, J.W. Ludlow and J. Dorrington. "*Steroidogenesis-Inducing Protein, Isolated from Human Ovarian Follicular Fluid, Is a Potent Mitogen for Cell Lines Derived from Ovarian Surface Epithelial Carcinomas.*" Gynecologic Oncology 66:501-508, 1997

3. Krtolica, A., N. Krucher and J.W. Ludlow. "*Hypoxia-Induced pRB Hypophosphorylation Results From Downregulation of CDK and Upregulation of PP1 Activities.*" Oncogene 17: 2295-2304, 1998.

   This paper describes molecular mechanisms involved in hypoxia-induced cell cycle arrest. We show that hypoxia-induced arrest is accompanied by a decrease in pRB-directed CDK4 and CDK2 activities, lower cyclin D and E protein levels, and by an increase in p27 protein abundance and its increased association with cyclin E-CDK2 complexes. Furthermore, hypoxia increases PP1-mediated pRB dephosphorylation. These data reveal that decreased pRB-directed cyclin/CDK activity and increased pRB-directed phosphatase activity contribute towards inducing and maintaining pRB in its hypophosphorylated, growth suppressive state during hypoxia.

4. Kruchrer, N., A. Krtolica and J.W. Ludlow. "*Mitogenic Activity of Steroidogenesis-Inducing Protein (SIP) During Hypoxic Stress of Human Ovarian Carcinoma Cells.*" Cancer Letters 133(2): 205-14, 1998.

5. Krtolica, A., N. Krucher and J. W. Ludlow. "*Molecular Analysis of Selected Cell Cycle Regulatory Proteins during Normoxic and Hypoxic Maintenance of Human Ovarian Carcinoma Cells.*" British Journal of Cancer 80(12): 1875-83, 1999.

6. Genbacev, O., A. Krtolica, W. Kaelin, and S. Fisher. "*Human Cytotrophoblast Expression of the von Hippel-Lindau Protein is Downregulated During Uterine Invasion In Situ and Upregulated by Hypoxia In Vitro.*" Dev. Biol. 233(2): 526-36, 2001.

7. Parrinello, S., C.Q. Lin, K. Murata, Y. Itahana, J. Singh, A. Krtolica, J. Campisi and P-Y. Desprez: *"Id-1, ITF-2 and Id-2 comprise a network of helix-loop-helix proteins that regulate mammary epithelial cell proliferation, differentiation and apoptosis."* JBC published August 9, 2001.

8. Krtolica, A., S. Parrinello, S. Lockett, P.-Y. Desprez and J. Campisi. "*Senescent Fibroblasts Promote Epithelial Cell Growth and Tumorigenesis, Linking Cancer and Aging.*" PNAS *in press.*

   We showed that senescent human fibroblasts stimulate premalignant, but not normal, epithelial cells to proliferate in culture and form tumors in mice. In culture, the growth stimulation was evident when senescent cells comprised only 10% of the fibroblast population, and was equally robust whether senescence was induced by replicative exhaustion, oncogenic RAS, $p14^{ARF}$ overexpression or hydrogen peroxide. Moreover, the growth stimulation was due at least in part to soluble and insoluble factors secreted by senescent cells. In mice, senescent fibroblasts, much more than presenescent fibroblasts, caused premalignant and malignant epithelial cells to form tumors. Our findings suggest that, although cellular senescence may suppress tumorigenesis early in life, it may promote cancer in aged organisms, suggesting it is an example of evolutionary antagonistic pleiotropy.

Poster Presentations

1. Krtolica, A. and J.W. Ludlow. *"Influence of Hypoxic Stress on Invasive Properties of Human Ovarian Carcinoma Cells."* American Society for Cell Biology Annual Meeting. December 1994. San Francisco, CA.
2. Huflejt, M. E., A. Krtolica, J.W. Ludlow, H. Leffler and S. H. Barondes. *"Characterization of Galectins in Human Ovarian Cancer Cell Lines."* American Society for Cell Biology Annual Meeting. December 1994. San Francisco, CA.
3. Krucher, N., A. Krtolica and J.W. Ludlow. *"Effect of Hypoxia on the Phosphorylation State of the Retinoblastoma Gene Product (pRB)."* American Society for Cell Biology Annual Meeting. December 1996. San Francisco, CA.
4. Krtolica, A., D. Yip, G. Dimri, P. Y. Desprez, and J. Campisi. *"The Double-edged Sword of Replicative Senescence: Senescent Fibroblasts Stimulate Pre-malignant Epithelial Cell Growth."* Young Investigator Award recipient. American Association for Cancer Research Annual Meeting. April 2000. San Francisco, CA.

Invited Seminar Presentations

1. University of California San Francisco, San Francisco, CA: *"Models for studying role of altered stroma in tumorigenesis."* February 2001.
2. National Cancer Institute, NIH, Bethesda, Maryland: *"Pay-off time: cellular senescence stimulates tumorigenesis."* October 2000.
3. University of Belgrade, Belgrade, Yugoslavia: *"Regulation of the cell cycle and hypoxia."* June 1997.
4. Stanford University, Palo Alto, CA: "*Hypoxia and the cell cycle.*" April 1997.
5. University of California San Francisco, San Francisco, CA: *"The effects of hypoxia on cell proliferation."* December 1996.
6. University of Rochester Cancer Center Grand Rounds, Rochester, NY: *"Influence of hypoxia on ovarian carcinoma invasiveness and proliferation."* April 1995.

Funding

1998 - present DOD-BCRP Post-Doctoral Fellowship

CA-TBRP 2001 Idea Award: *"Tobacco-altered stromal cells may promote carcinogenesis" ($75,000, 1.5 years, pending)*

DOD-BCRP 2001 IDEA Award: *"Aging, mutations and breast carcinogenesis" ($475,000, 3 years, pending)*

DOD-BCRP 2001 Career Development Award: *"Aging, mutations and breast carcinogenesis" ($420,000, 4 years, pending)*

Education

Graduate studies at the Department of Biochemistry ***1992 - 1997***
University of Rochester,
Rochester, NY

- *M.S. in Biochemistry* *1995*
- *Ph.D. in Biochemistry* *1997*

Biochemistry Major Undergraduate Studies ***1988-1992***
University of Belgrade,
Belgrade, Yugoslavia

- *B.S. in Biochemistry* *1992*

Teaching Experience

Teaching Assistantship and Tutoring ***1994-1997***

Teaching assistant and tutor for two graduate level courses at the University of Rochester Department of Biochemistry (Structure and Function of Macromolecules and Advanced Biochemistry).

1997-present

Supervised multiple undergraduate and graduate students in performing their research projects in the laboratory.

Computer Skills

Include: use of word processing, data processing (spreadsheet), data presentation, statistical analysis, image processing and analysis programs in both IBM compatible and Macintosh environments.

Awards Received

2000	AACR Young Investigator Award
1998 - present	Breast Cancer Army Post-Doctoral Fellowship
1995 –1996	Elon Huntington Hooker University Graduate Fellowship
1994	Teaching Award
1992	The Best Student of the Class '92 Award

Languages

English, Russian, Serbo-Croat

References

Dr Judith Campisi *(supervisor)*, Senior Scientist, Life Sciences Division, Lawrence Berkeley National Laboratory, 1 Cyclotron Road, Mailstop 84-171, Berkeley, CA 94720, 510 486-4545; Email: jcampisi@lbl.gov

Dr. Susan Fisher, Profesor, Department of Stomatology, Box 0512, HSW 604, University of California San Francisco, 513 Parnassus, San Francisco, CA 94143, phone: (415) 476-5297, Email: sfisher@cgl.ucsf.edu

Dr. Pierre Desprez, Staff Scientist, Geraldine Brush Cancer Research Institute, California Pacific Medical Center, 2333 Buchanan, San Francisco, CA 94115, phone: (415) 561-1760, Email: pdesprez@cooper.cpmc.org

Dr. John Ludlow *(thesis advisor)*, Director, Cell Therapy Program, Incara Pharmaceuticals, P.O. Box 14287, Research Triangle Park, North Carolina 27709, phone: (919) 558-1928, Email: jludlow@incara.com